Relation between CYP17 Polymorphism and Hyperandrogenemia in Polycystic Ovarian Syndrome

Hubungan Polimorfisme CYP17 dengan Kejadian Hiperandrogenemia pada Sindrom Ovarium Polikistik

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Abstract

Objectives: To find 5-untranslated region polymorphism of CYP17 gene and its connection with hyperandrogenemia in polycystic ovarian syndrome.

Methods: A cross sectional descriptive study with consecutive random sampling method. Body mass index, ovarian morphology by ultrasonography, fasting insulin level, fasting blood glucose level, 17-hydroxyprogesterone level, total testosterone level, serum hormone binding globulin level, and CYP17 gene polymorphism in 45 subject with PCOs and 45 control subject who attend Yasmin clinic of Cipto Mangunkusumo General Hospital with menstruation problems were measured. CYP17 gene polymorphism was evaluated using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method with MspA1 restriction enzyme.

Results: In PCOs group, the genotype distribution were; 11.1% subject with genotype CC, 71.1% subject with genotype TC and 17.8% subject was wild type TT. In non PCOs group, the genotype distribution for CC, TC and TT respectively were 13.3%, 46.7% and 40%. There was significant difference between both group in distribution of TC and TT genotype, with p value 0.013. Frequencies of allele c and allele t in PCOs group were 47% and 53%. In non PCOs group, frequency of allele c and t were 37% and 63%. There were tendency for higher frequency of allele c in the PCOs group but the difference was not statistically significant. Median FAI value for genotype CC homozygote, TC heterozygote and TT homozygote in PCOs group respectively were; 6.82 (6.07 - 8.23); 5.59 (0.25 -21.45) and 4.74 (3.48 - 8.88). There was tendency for increase FAI value in PCOs group corresponds to variant allele, but the result was not statistically different.

Conclusion: There were higher proportion of CC homozygote and TC heterozygote genotype in PCOs patient with tendency of increasing FAI value.

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Keyword: polycystic ovarian syndrome, free androgen index, CYP17 polymorphism

Abstrak

Tujuan: Mengetahui prevalensi polimorfisme CYP17 pada 5-UTR dan hubungan antara polimorfisme CYP17 dengan hiperandrogenemia pada sindrom ovarium polikistik.

Metode: Penelitian deskriptif dengan studi potong lintang. Indeks massa tubuh, penilaian ovarium, kadar hormon LH/FSH, insulin puasa, gula darah puasa, 17 hidroksi progesteron, testosteron total, SHBG dan polimorfisme CYP17 dinilai pada 45 subjek SOPK dan 45 subjek kontrol yang datang ke klinik Yasmin RSCM dengan keluhan haid. Pemeriksaan polimorfisme CYP17 dilakukan dengan PCR RFLP dengan menggunakan enzim restriksi MspA1.

Hasil: Pada kelompok SOPK didapatkan genotip CC pada 5 (11,1%) subjek, genotip TC 32 (71,1%) subjek dan genotip TT pada 8 (17,8%) subjek. Pada kelompok non SOPK, jumlah subjek dengan genotip CC, TC dan TT berturut-turut 6 (13,3%), 21 (46,7%), dan 18 (40%). Pada perbandingan antara kelompok homozigot CC dengan wild type TT didapatkan hasil yang tidak bermakna secara statistik dengan nilai p 0,392. Hasil yang bermakna dilihat dari perbandingan antara kelompok heterozigot TC dengan wild type TT, dengan nilai p 0,013. Pada kelompok SOPK, ditemukan frekuensi alel c sebesar 47% dan frekuensi alel t 53%. Sedangkan pada kelompok non SOPK frekuensi alel c adalah 37% dan frekuensi alel t 63%. Terdapat kecenderungan peningkatan frekuensi alel c pada kelompok SOPK dibandingkan dengan kelompok non SOPK namun hasil tersebut tidak bermakna secara statistik dengan nilai p 0,1. Didapatkan hasil median kadar FAI dari kelompok homozigot CC, heterozigot TC dan wild type TT SOPK berturut-turut adalah 6,82 (6,07 - 8,23); 5,59 (0,25 - 21,45) dan 4,74 (3,48 - 8,88). Terdapat kecenderungan peningkatan nilai FAI pada kelompok genotip homozigot CC dan heterozigot TC, namun hasilnya tidak bermakna secara statistik dengan nilai p 0,468.

Kesimpulan: Proporsi genotip CC dan TC lebih tinggi pada pasien SOPK dan terdapat kecenderungan peningkatan nilai FAI pada kelompok homozigot XX dan heterozigot TC, namun hasil tersebut tidak bermakna secara statistik.

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Kata kunci: sindrom ovarium polikistik, free androgen index, polimorfisme CYP17

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INTRODUCTION

Since first introduced by Stein and Leventhal in 1935, Polycystic Ovarian Syndrome (PCOs) is one of the leading endocrinology problem found in women and caused infertility due to anovulation.[1]

Polycystic ovaries were found in 73% infertility cases with anovulation, in 37% women with amenorrhea and in 90% women with oligomenorrhea.[2] To find the etiology and understand the pathophysiology of PCOs might help us to figure out the most appropriate management.

The diagnosis of PCOs was confirmed if there were 2 out of 3 conditions below; oligomenorrhea or anovulation, clinical sign or biochemistry of hyperandrogenemia and polycystic ovary seen by ultrasonography.[3] The prevalence of PCOs using this criteria was 22% with incidence in the reproductive age women is 1 out of 12.[4]

PCOs was a cohort of signs and symptoms which lead to reproductive, endocrinology and metabolic disturbance. The patophysiology was considered multifactorial and polygenic.[5] From many studies, the leading hypothesis were disturbance in insulin secretion and action regulator gene; disturbance in folliculogenesis regulator gene (gonadotropin regulator gene); weight and energy regulator gene; and androgen secretion and action regulator gene. Any disturbance in those genes will cause clinical manifestation of PCOs.[3,6]

Even though considered caused by multifactorial etiology, one of the patophysiology and included into the diagnostic criteria is hyperandrogenemia.[2,7] As stated before disruption in androgen synthesis gene might result in androgen hormone level abnormality. Many gene candidates were thought to be responsible in regulation of androgen biosynthesis. Urbanek et al found 8 genes related to hyperandrogenemia in PCOs, one of them is CYP17 gene.[8] Rate of androgen biosynthesis was depend on 17-hydroxylase/17,20 lyase enzyme activity (also known as CYP17). This enzyme is family of cytocrome P450, coded by CYP17 gene that located in chromosome 10q24,3. CYP17 acted through 17α-hydroxylase activity will convert 17α-hydroxypregnenolone into cortisol. When 17α-hydroxylase and 17,20-lyase were activated, dehydroepiandrosterone (DHEA) will be produced. The DHEA will then be converted into androstenedione by 3ßHSD II enzyme activity. Androstenedione will be converted to testosterone and estradiol by isoenzyme 17ß-hydroxysteroid dehydrogenase and aromatase.[6,9,10] Through this androgen biosynthesis pathway, CYP17 considered as having an important role.

Study in ovarian theca cell found an enhancement of expression and activity of CYP17 gene in PCOs women. Numerous factors affecting CYP17 gene activity in PCOs, one was stated by Carey at al; there was variant allele in CYP17 gene.[8]

Until recently, one of CYP17 variant allele that has been found is single nucleotide polymorphism T→C in 5'-untranslated region (5-UTR). This polymorphism will create new promoter, enhance CYP17 activity and consecutively increase the androgen biosynthesis rate. Polymorphism in 5-UTR will create new variant with genotype TC heterozygote and CC homozygote.[6] Diamanti et al shows an increased total testosterone level in TC heterozygote compared with the wild type genotype.

Previous study linked CYP17 polymorphism with hyperandrogenemia using androstenedione or total cholesterol for measuring hyperandrogenemia. Unfortunately, total testosterone level could not show testosterone bioavailability due to the influence of serum hormone binding globulin (SHBG) level. Free androgen index (FAI) could be more accurate in showing free testosterone level in blood. This FAI was calculated from total testosteron divided by SHBG. This study will evaluate hyperandrogenemia with the parameter of an increased FAI due to CYP17 polymorphism.

METHODS

The study was conducted after FKUI ethical commission approval and the sample was collected from July to October 2010. This was a descriptive with comparative cross sectional study.

Subject was chosen with consecutive random sampling. The study population were patients coming to YASMIN clinic at Dr. Cipto Mangunkusumo General Hospital with the diagnosis of PCOs and as control are patients with other problems. The sample was chosen with simple random sampling. Patient with hyperandrogenemia due to congenital adrenal hyperplasia were excluded.

Calculated sample size using 0.15 as d constanta was 45 subject. Prior to signing the consent, subjects were informed. Interview was conducted regarding the basic data, followed by physical examination including BMI, and transvaginal ultrasonography.

Sample collection for laboratory examination (LH, FSH, fasting blood glucose, fasting insulin, total cholesterol, SHBG and CYP17 polymorphism) was conducted in day 3 - 5 of cycle in patient with regular cycle and in any days for patient with irregular menstruation.

Isolation of DNA genome for genotype analysis was taken from peripheral blood sample using DNA genomarker kit extractor (Blossom, Taiwan). Primers used in DNA amplification were forward primer 5'-CAT-TCG-CAC-TCT-GGA-GTC-3' and reverse primer 3'-AGG-CTC-TTG-GGG-TAC-TTG-5' (Eurogentec AIT).

Five µl of DNA genome (extracted from leucocyte using QIAamp DNA Mini and Bllod Mini Kit) added to 20 µm of every primers, 1.5 mM $MgCl_2$, 0.2 mm deoxyribonucleotide triphosphate and 0,1 U AmpliTaq polymerase DNA (Invitrogen.Cat.No.10966-018).

ThermalCycler (PE2400) was used for amplification with total 35 cycle in heat condition 94°C for 3 minutes, 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute and 72°C for 5 minutes.

Amplicon was digested for one hour using 0.5 µl MspA1 I (AIT). Restriction fragment lenght polymorphism (RFLP) analysis was conducted using electrophoresis in 2% agarose gel. The endonuclear genotype is considered as "T" if there was no restriction site and stated as "C" when there was a restriction site.

Amplicon with 414-bp was restricted by MspA1 enzyme to two band sized 290-bp and 124-bp. Homozygote TT/wild type will be seen as single band sized 414-bp. Homozygote CC will be seen as dual band sized 290-bp and 124-bp. While the heterozygote TC will be seen as three bands sized 414-bp, 290-bp and 124-bp.

Genotype frequencies and allele in both group and in between different genotype will be calculated and compared.

RESULT

Sample collection was conducted from July 2010 to October 2010. Research sample consists of 45 responders for each group.

Mean age of each group were comparable. Medical data of nutritional status (body mass index), insulin resistance, testosteron level and free androgen index from both group were compared.

From PCOs group 60% of the subject were considered as obese. Insulin resistance was found in 67% subject in PCOs group while in non PCOs group, only 12% were considered having insulin resistance.

Adrogen profile of both group were measured using testosteron level and FAI. (see Table 1)

Table 1.   Androgen profile in both group.

Androgen level	PCOs	Non PCOs	p value
Testosterone (nmol/l)	130,77 (6,95 - 489,42)	65,90 (9,91 - 199,39)	0,000
FAI	6,07 (0,25 - 21,45)	1,87 (0,24 - 5,00)	0,000

Median total testosterone level and FAI were higher in PCOs group, and the results were statistically significant with p value 0.000.

CYP17 Genotype, Molecular Analysis

The amplicon from PCR has sequence as showed in Figure 1.

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AGG CTC TTG GGG TAC TTG GCA CCA GGG CAC CTT CTC TTG GGC CAA AAC
AAA TAA GCT AGG GTA AGC AGC AAG AGA GCC ACG AGC TCC CAC ATG GTG
GCT GGG TGC CGG CAG GCA AGA TAG ACA GCA GTG GAG TAG AAG AGC TGT
GGC AAC TCT AGG GCA CAA GGA GGC CTT TTA AAG GGC TAC CCT GAT CTT
CAC CTT GAC TTT  GTG TTA TCT CTT GCC TTG TGG AAA GAT TCT CCT GGA
GCC CAG CCA GGC CTG AGC TCA TAT CCA GAA GGG AGA GAG GCG GTG GGA
GTG AAG GCC TCC TCA AGG GCT GGC TCA ACT CCA GGG CAA ACC TCC GGA
GGA GGA GCT AGG TAA GGG AGG TCA GTT GAT CAC CCT CTG AGG AGC TCC
CCA TGC TTG AAT GAC TCC AGA GTG CGA ATG
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Figure 1.   Amplicon from PCR.

The arrow was marking the restriction site that would be recognized by MspA1 restriction enzyme.

In DNA sample with CYP17 polymorphism, the sequens CAG CAG will change into CAG CGG and will be recognized by MspA1 I restriction enzyme. Using electrophoresis in agarose 2% gel, the genotype of DNA sample will be known. Sample with TT homozygote genotype will be seen as one band; sample with CC homozygote genotype will be seen having dual band while TC heterozygote genotype shown as three bands. (see Figure 2)

Sample number 6, 12, 13, 20 and 68 are 414-bp DNA showing TT homozygote genotype. Sample number 11 and 24 are 290-bp and 124-bp DNA fragment showing CC homozygote genotype and the rest are TC heterozygote genotype consist of 414-bp, 290-bp and 124-bp DNA fragment.

Result for genotype and allele frequencies in both group were mentioned below.

In PCOs group the genotype were 11.1% was CC homozygote, 71.1% was TC heterozygote and 17.8%

was TT homozygote. While in non PCOs group the genotypes for CC, TC and TT consecutively were 13.3%, 46.7% and 40%. A statistically significant result (p value 0.013) was shown when we compared the heterozygote TC and wild type TT between both group, while result of comparison from homozygote CC and wild type TT showed no significant difference (p value 0.392).
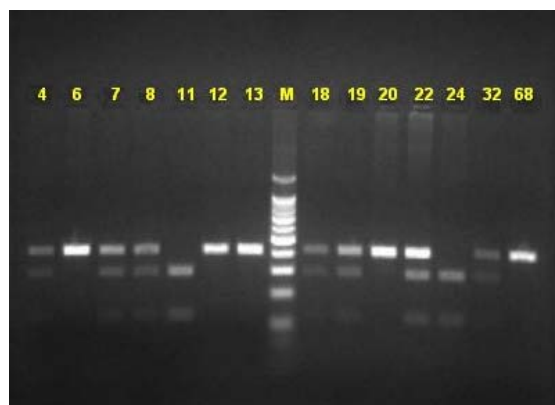

Figure 2.   Detection of 5'-UTR CYP17 polymorphism with PCR-RFLP using MspA1 I restriction enzyme.

For allele frequencies; in PCOs group allele c was found in 47% subject and allele t frequency was 53%, while in non PCOs group frequency of allele c and t consecutively were 37% and 63%. There were tendency of increasing allele c frequency in PCOs group but the result was not statistically significant (p value 0.1).

Relationship between CYP17 genotype and androgene level were shown in Table 2.

Total testosterone level tend to increase in CC homozygote compared with TC heterozygote and TT homozygote. Median testosterone level were 10.84% increased in CC homozygote compared with TT homozygote.

Table 2.   Relation of CYP17 genotype in PCOs group with total testosterone level and FAI.

Genotype	PCOs n	FAI	Testosterone nmol/l
CC	5	6,82 (6,07 - 8,23)	145,92 (129,65 - 489,42)
TC	32	5,59 (0,25 - 21,45)	120,23 (6,95 - 282,49)
TT	8	4,74 (3,48 - 8,88)	131,65 (83,97 - 276)
p		0,468	0,394

Hyperandrogenemia also assessed using FAI. The FAI also tend to be increased in CC homozygote and TC heterozygote group but the result was not statistically different.

DISCUSSION

The main patophysiology for PCOs was still a mystery need to be solved. The complex clinical signs

and symptoms needs to be explained by understanding the different patophysiological mechanisms.

Four main theories discussed worldwide were[1] androgene synthesis defect result in increasing ovarian androgene hormone production;[2] disturbance in insulin secretion and action caused hyperinsulinemia and insulin resistance;[3] primary neuroendocrine defect caused increasing LH frequency and amplitude; and[4] cortisol metabolism changes caused adrenal androgene production.[11]

Ovarian steroid biosynthesis depend on several chemical reaction, started with cholesterol translocation to inner membrane of mitochondria regulated by steroidogenic acute regulatory protein and continued with steroidogenesis process in ovarian theca cell. Numerous genes were involved in regulation of steroid biosynthesis, such as CYP17, CYP11A (P450 cholesterol side chain cleavage enzyme) and 3ß-hydroxysteroid dehydrogenase.[6]

CYP17 gene is located in chromosome 10q24.3 which coded cytocrome enzyme P450c17α with 17α-hydroxylase and 17,20-lyase activity. This enzyme will convert pregnenolone and progesterone into 17-hydroxypregnenolone and 17-hydroxyprogesterone and continued with convertion of both form of steroid into dehydroepiandrosterone and androstenedione.[1] Previous study in ovarian theca cell of PCOs patient found an increased CYP17 gene transcription and cAMP dependent. Other study found a single nucleotide polymorphism T/C in promoter region resulting in increase of CYP17 activity.[8]

Carey et al in 1994 found increasing risk to have PCOs in person with variant allele. This variant allele connected with androgen hormone increasing.[8]

Until now, 3 CYP17 gene polymorphism already found; T/C mutation in 5'-UTR, G/A mutation in promoter region of nucleotide 1951 and T/A mutation in 5471 nucleotide.

Polymorphism in 5'-UTR create variant allele with new promoter. Amount of promoter correlate with promoter activity. So this variant allele were considered to cause transcriptional increase. Increasing transcription will cause increasing activity and continued with increasing androgen level in the ovary with the end result was ceased of follicle maturation.[12]

Insulin resistance prevalence were higher in the PCOs group. Peripheral insulin resistance will increase the plasma insulin level. Insulin will bound to IGF-1 and together increase theca cell androgen production under the influence of luteinizing hormone. Insulin will also decrease liver SHBG production, caused increasing testosteron bioavailability due to increasing plasma free testosterone level.

Hyperandrogenemia was classified with total testosterone level and FAI. Total testosterone level and FAI in PCOs group were higher than control group and the difference was statistically significant.

For 5'-UTR CYP17 polymorphism; in this study we found higher variant allele frequency among the PCOs group. The allele c frequency were 10% higher in PCOs group (47% to 37%). TC heterozygote genotype was found in 71% subject in PCOs group and CC homozygote genotype frequency was 11%.

Different result coming from different population.

CC homozygote frequency were higher in population with East Asian origin. Study in Singaporean, Taiwanese, Asian ethnic or India-Pakistan ethnic who lived in Canada showed CC homozygote frequency 32%. Smaller frequency were found among white skin population in North America, Europe and Australia; with CC homozygote frequency were 14%. In Russia, frequency for CC homozygote were 23 - 25%. One study in Nigeria, Africa found 9% CC homozygote frequency.[12]

In this sudy we compare the frequency of CC homozygote in both groups; frequency of TC heterozygote and CC homozygote were lower in non PCOs group. This shows tendency for varian gene with T/C polymorphism caused increase androgen synthesis.

Data about subject's ethnic should be included in genetic study. Indonesia is a country with many ethnical diversity with different characteristics. Many ways to classify ethnic groups were used. One of them is differentiating ethnic group based on parental origin; with austronesia if the patient came from Java, Borneo and Bali; Wallace if the patient came from Sulawesi and Sumbawa; and Papua for subjects from Papua. Limitation of this study is that we did not include ethnic group in the data.

In this study we found that variant allele will increase risk for having PCOs. Results from this study are consistent with many previous studies. We are using FAI to measured androgene hormonal status. This index shows testosterone bioavailability and spesifically shows androgene abnormality. In PCOs group the median FAI score were 20.6% higher in TC heterozygote group compared with TT homozygote and 29.4% higher in CC homozygote group compared with TT homozygote.

Other study using other measurement method to assess androgene status for subject with CYP17 polymorphism. Diamanti et al compared testosterone level in TT homozygote with heterozygote, with result 15% higher testosterone level in homozygote group.[13] Haiman et al compared testosterone, androstenedione, DHEA and DHEAS level between genotype and found; 6% higher of testosterone level (p = 0.40), 5% higher of androstenedione level (p = 0.47), 2% higher of DHEAS level (p = 0.82) and 7% higher of DHEA level (p = 0.47) in CC homozygote compared with the wild type.[14] Other study from Daneshman et al compared testosterone plasma level and androstenedione in follicle fluid, they found 2% higher testosterone level and 40% higher androstenedione level in TC heterozygote compared with TT homozygote.[15] Garcia-Closas also found higher mean androstenedione and testosterone level in CC homozygote compared with TC heterozygote and TT homozygote; androstenedione level were 0.3% higher in TC heterozygote compared with CC homozygote and testosterone level were 4% higher.[16]

Different result came from non PCOs group. The FAI in CC homozygote were lower than TT homozygote. We previously thought that CYP17 polymorphism in non PCOs group was not related with increasing androgen production. Many factors contributes in steroid metabolism.

First we thought about other mutation site in CYP gene. Until now we have already found 3 point of

mutation; T/C polymorphism in 5'-UTR region; G/A mutation in nucleotide 1951 or promoter region; and C/A mutation in nucleotide 5471.[12] Increasing activity of CYP17 enzyme might be caused by one of those mutation or if there were two mutations in the same individual. Whether one point of mutation is having higher influence than other need to be proven in following study.

The second factor is the presence of phosphorylation which influence CYP17 enzyme activity. Human CYP17 will through serine and treonine phosphorylation via cAMP-dependent protein kinase. CYP17 phosphorylation will increase 17.20-lyase activity and dephosphorylation will decrease it. Lin-Hua Zhang concluded that cAMP dependent mechanism in adrenal gland and other kinase dependent mechanism in ovarium will cause CYP17 phosphorylation. In addition, abnormality of LH secretion in PCOs patient may cause an impairment of serine phosphorylation. Serine phosphorylation in insulin receptor also cause insulin resistance.[17,18] Further studies are needed to explain the relationship between serine phosphorylation, CYP17 polymorphism and hyperandrogenemia.

Several other genes and enzymes take part in controlling steroid biosynthesis. Those agents are; Cytocrome P450scc or known as CYP11a which control the production and responsible for 17-hydroxyprogesterone conversion to 11-deoxycortisol; aromatase gene responsible for conversion of C19 steroid to C18 steroid.[19,20] Probability of mutation and interaction among those genes in causing impairment of steroid metabolism needs further studies.

Another explanation is a complexity of CYP17 promoter activity. In adrenal cortex, building protein structure started with stimulation of adrenocorticotropin (ACTH) to activate steroidogenic gene promoter. Some transcription factors were already known to cause disruption in promoter activity. Those are; steroidogenic factor-1 (SF-1), GATA-6 and sterol regulatory binding protein 1 (SREBP1).[21] Those transcription factors part in disrupting androgen biosynthesis also need to be explored further.

CONCLUSION

This study found a higher proportion of CC and TC genotype in PCOs patient and the tendency of increasing free androgen index level in patients with genotype CC homozygote and TC heterozygote.

Further study about interaction of CYP17 polymorphism with other gene and other factor that impaired CYP17 activity with end result androgen elevation should be conducted with a larger sample size and adding an ethnical analysis.

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